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(54) Title: CASPASE-14 POLYPEPTIDES

(57) Abstract

The present invention relates to a novel Caspase-14 protein (also called ERICE) which is a novel effector of apoptosis. In particular, isolated nucleic acid molecules are provided encoding the human Caspase-14 protein. Caspase-14 polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of Caspase-14 activity. Also provided are therapeutic methods for treating diseases and disorders associated with apoptosis.

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CASPASE-14 POLYPEPTIDES

Field of the Invention

The present invention relates to a novel effector of apoptosis. More specifically, isolated nucleic acid molecules are provided encoding a human Caspase-14 polypeptide, sometimes herein after referred to as "ERICE". Caspase-14 polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of Caspase-14 activity. Also provided are therapeutic methods for treating diseases and disorders associated with apoptosis.

Background of the Invention

The cell death machinery is conserved throughout evolution and is composed of activators, inhibitors, and effectors (Chinnaiyan, A.M. and Dixit, V.M., *Curr. Biol. 6*:555-562 (1996)). The effector arm of the cell death pathway is composed of a rapidly growing family of cysteine aspartate-specific proteases termed caspases (Alnemri, E.S., et al., *Cell 87*:171 (1996)). As implied by the name, these cysteine proteases cleave substrates following an aspartate residue (Alnemri, E.S., et al., *Cell 87*:171 (1996); Walker, N.P., et al., *Cell 78*:343-352 (1994)). Caspases are normally present as single polypeptide zymogens and contain an amino-terminal prodomain, and large and small catalytic subunits (Wilson, K.P., et al., *Nature 370*:270-274 (1994); Rotonda, J., et al., *Nat. Struct. Biol. 3*:619-625 (1996); Fraser, A. and Evan, G., *Cell 85*:781-784 (1996)). The two chain active enzyme (composed of the large and small subunits) is obtained following proteolytic processing at internal Asp residues (Wilson, K.P., et al., *Nature 370*:270-274 (1994); Rotonda, J., et al., *Nat. Struct. Biol. 3*:619-625 (1996); Fraser, A. and Evan, G., *Cell 85*:781-784 (1996)). As such, caspases are capable of activating each other in a manner analogous to zymogen activation that is observed in the coagulation cascade (Boldin, M.P., et al., *Cell 85*:805-815 (1996)). The identification of FLICE and Mch4/FLICE2 as receptor associated caspases suggested a surprisingly direct mechanism for activation of the death pathway by the cytotoxic receptors CD-95 and TNFR-1 (Boldin, M.P., et al., *Cell 85*:805-815 (1996); Muzio, M., et al., *Cell 85*:817-827 (1996); Vincenz, C. and Dixit, V.M., *J. Biol. Chem. 272*:6578-6583 (1997); Chinnaiyan, A.M., et al., *Cell 81*:505-512 (1995)). Upon activation, both receptors use their death domains to bind the corresponding domain in the adaptor molecule

FADD (Fas-associated death domain protein) (Muzio, M., et al., *Cell 85*:817-827 (1996); Vincenz, C. and Dixit, V.M., *J. Biol. Chem. 272*:6578-6583 (1997); Chinnaiyan, A.M., et al., *Cell 81*:505-512 (1995)). Dominant negative versions of FADD that lack the N-terminal segment but still retain the death domain potently inhibit both CD-95 and TNFR-1 induced apoptosis (Chinnaiyan, A.M., et al., *J. Biol. Chem. 271*:4961-4965 (1996); Muzio, M., et al.. *J. Biol. Chem. 272*:2952-2956 (1997)). Given the importance of the N-terminal segment in engaging the death pathway, it has been termed the death effector domain (DED) (Chinnaiyan, A.M., et al., *J. Biol. Chem. 271*:4961-4965 (1996)).

Remarkably, the DED is present within the prodomain of FLICE and Mch4/FLICE2 and mutagenesis studies suggest that a homophilic interaction between the DED of FADD and the corresponding domain in FLICE or Mch4/FLICE2 is responsible for the recruitment of these proteases to the CD-95 and TNFR-1 signaling complexes (Muzio, M., et al., *Cell 85*:817-827 (1996); Vincenz, C. and Dixit, V.M., *J. Biol. Chem. 272*:6578-6583 (1997); Chinnaiyan, A.M., et al., *Cell 81*:505-512 (1995); Chinnaiyan, A.M., et al., *J. Biol. Chem. 271*:4961-4965 (1996)). Taken together, these data are consistent with FLICE and Mch4/FLICE2 being apical enzymes that initiate precipitous proteolytic processing of downstream caspases resulting in apoptosis (Boldin, M.P., et al., *Cell 85*:805-815 (1996); Srinivasula, S.M., et al., *PNAS 93*:14486-14491 (1996); Fernandes-Alnemri, T., et al., *PNAS 93*:7464-7469 (1996); Henkart, P.A., *Immunity 4*:195-201 (1996)). A number of viral gene products antagonize CD-95 and TNFR-1 mediated killing as a means to persist in the infected host (Shen, Y. and Shenk, T.S., *Current Opinion in Genetics and Development 5*:105-111 (1995)). The poxvirus encoded serpin CrmA and baculovirus gene product p35 are direct caspase inhibitors (Walker, N.P., et al., *Cell 78*:343-352 (1994)). In contrast, the molluscum contagiosum virus protein MC159 and the equine herpes virus protein E8 encode DED-containing decoy molecules that bind to either FADD (MC159) or FLICE (E8) and disrupt assembly of the receptor signaling complex, thereby abrogating the death signal (Hu, S., et al., *J. Biol. Chem. 272*:9621-9624 (1997); Bertin, J., et al., *PNAS 94*:1172-1176 (1997); Thome, M., et al., *Nature 386*:527-521 (1997)). The existence of these viral inhibitors has raised the question of whether functionally equivalent molecules are encoded in the mammalian genome.

There is a need for factors, such as the polypeptides of the present invention, that are useful for effecting or inhibiting apoptosis for therapeutic purposes, for example, in the treatment of Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, septic shock, sepsis, stroke, CNS inflammation, osteoporosis, ischemia, reperfusion injury, cell death associated with cardiovascular disease, polycystic kidney disease, apoptosis of endothelial cells in cardiovascular disease, degenerative liver disease, MS and head injury damage. There is a need, therefore, for the identification and characterization of such factors that are effectors or inhibitors of apoptosis,

such as Caspase-14 polypeptides of the present invention, which can play a role in preventing, ameliorating or correcting the diseases and disorders associated with apoptosis.

Summary of the Invention

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the Caspase-14 polypeptide having the amino acid sequence shown in SEQ ID NO:2 or the amino acid sequence encoded by the human cDNA in the clone deposited as American Type Culture Collection ("ATCC") Deposit No.209039 on May 15, 1997. The ATCC is located at 10801 University Boulevard, Manassas, Virginia 20110-2209.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of Caspase-14 polypeptides or peptides by recombinant techniques.

The invention further provides an isolated Caspase-14 polypeptide having an amino acid sequence encoded by the polynucleotides described herein. The present invention also provides a screening method for identifying compounds capable of enhancing or inhibiting a cellular response induced by the Caspase-14, which involves contacting cells which express the Caspase-14 with the candidate compound, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made in absence of the candidate compound; whereby, an increased cellular response over the standard indicates that the compound is an agonist and a decreased cellular response over the standard indicates that the compound is an antagonist.

In another aspect, a screening assay for agonists and antagonists is provided which involves determining the effect a candidate compound has on Caspase-14 binding to the TNFR-1, TRAIL, or CD-95 receptor. In particular, the method involves contacting the TNFR-1, TRAIL or CD-95 receptor with a Caspase-14 polypeptide and a candidate compound and determining whether Caspase-14 polypeptide binding to the TNFR-1, TRAIL or CD-95 receptor is increased or decreased due to the presence of the candidate compound.

An additional aspect of the invention is related to a method for treating an individual in need of an increased level of Caspase-14 activity in the body comprising administering to such an individual a composition comprising a therapeutically effective amount of an isolated Caspase-14 polypeptide of the invention or an agonist thereof.

A still further aspect of the invention is related to a method for treating an individual in need of a decreased level of Caspase-14 activity in the body comprising, administering to such an individual a composition comprising a therapeutically effective amount of a Caspase-14 antagonist.

Brief Description of the Figures

Figure 1 shows the nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of Caspase-14 (encoded by cDNA clone HFJAB36 deposited with the ATCC as Deposit No. 209039). The protein has 377 amino acid residues and a deduced molecular weight of about 43.3 kDa.

Figure 2 shows an analysis of the CASPASE-14 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues from about 35 to about 71, about 79 to about 99, about 110 to about 138, about 173 to about 202, about 221 to about 250, about 259 to about 297, about 305 to about 318 and from about 343 to about 370 in Figure 1 (SEQ ID NO:2) correspond to the shown highly antigenic regions of the CASPASE-14 protein.

Detailed Description

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the Caspase-14 (or ERICE) polypeptide having the amino acid sequence shown in SEQ ID NO:2, which was determined by sequencing a cloned cDNA. The acronym ERICE stands for Evolutionarily Related Interleukin-1β Converting Enzyme. The Caspase-14 protein of the present invention shares sequence homology with other members of the Caspase family including, Caspase-1, Caspase-4, Caspase-5, Ice-3 Mouse, Caspase-3, Caspase-6, Caspase-7, Caspase-8, Caspase-10, Caspase-2 and Caspase-9. The nucleotide sequence shown in SEQ ID NO:1 was obtained by sequencing a cDNA clone (HFJAB36), which was deposited on May 15, 1997 at the American Type Culture Collection, 10801 University Blvd., Manassas Virginia 20110, and given accession number 209039. The deposited clone is inserted in the pBluescript SK(-) plasmid (Stratagene, La Jolla, CA).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at

least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Using the information provided herein, such as the nucleotide sequence in SEQ ID NO: 1, a nucleic acid molecule of the present invention encoding a Caspase-14 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule described in SEQ ID NO:1 was discovered in a cDNA library derived from human skin fibroblasts. The determined nucleotide sequence of the Caspase-14 cDNA of SEQ ID NO:1 contains an open reading frame encoding a protein of about 377 amino acid residues and a deduced molecular weight of about 43.3 kDa. The Caspase-14 protein shown in SEQ ID NO:2 is most closely related to Caspase-4 showing overall about 75% similar to Caspase-4. Caspase-14 shows strong similarity to many members of the Caspase family and, in particular, the QAC(R/Q/G)G motif conserved in all caspases is conserved in Caspase-14 (residues 256-260 in SEQ ID NO:2).

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) shown in SEQ ID NO:1; DNA molecules comprising the coding sequence for the Caspase-14 protein; and DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic

code, still encode the Caspase-14 protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the Caspase-14 polypeptide having an amino acid sequence as encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 209039 on May 15, 1997. In a further embodiment, nucleic acid molecules are provided encoding the Caspase-14 polypeptide or the full-length Caspase-14 polypeptide lacking the N-terminal methionine. The invention also provides an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 or the nucleotide sequence of the Caspase-14 cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping, by *in situ* hybridization with chromosomes, and for detecting expression of the Caspase-14 gene in human tissue, for instance, by Northern blot analysis.

Caspase-14 is believed to be produced as a proprotein which consists of a prodomain (residues 1 to 95 in SEQ ID NO:2), a large subunit domain (residues 96 to 277 in SEQ ID NO:2), and a small subunit domain (residues 289 to 377 in SEQ ID NO:2. Caspase-14 is believed to be processed by caspase-8 and that the large and small subunit domains form a heterodimer which is the active fragment. The heterodimeric fragment is believed to be reproducible in vitro.

The present invention includes polypeptides comprising the following conserved domains: (a) the large subunit domain of about 175 amino acids (residues 96 to 270 in SEQ ID NO:2); and (b) the predicted small subunit domain of about 89 amino acids (residues 289 to 377 in SEQ ID NO:2). Also provided are polynucleotides encoding such polypeptides.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNA or the nucleotide sequence shown in SEQ ID NO:1 is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments 50-600 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNA or as shown in SEQ ID NO:1. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited cDNA or the nucleotide sequence as shown in SEQ ID NO:1.

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the CASPASE-14 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 35 to about 71, from about 79 to about 99, from about 110 to about 138, from about 173 to about 202, from about 221 to about 250, from about

259 to about 297, from about 305 to about 318 and from about 343 to about 370 in SEQ ID NO:2. The inventors have determined that the above polypeptide fragments are antigenic regions of the CASPASE-14 protein. Methods for determining other such epitope-bearing portions of the CASPASE-14 protein are described in detail below.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clone contained in ATCC Deposit 209039. By "stringent hybridization conditions" is intended overnight incubation at 42 C in a solution comprising: 50% formamide, 5x SSC (750mM NaCl, 75mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65 C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNAs or the nucleotide sequence as shown in SEQ ID NO:1). Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3 terminal poly(A) tract of the Caspase-14 cDNA shown in SEQ ID NO:1), or to a complementary stretch of T (or U) resides, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode a Caspase-14 polypeptide may include, but are not limited to those encoding the amino acid sequence of the polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as those encoding a secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In

certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al., Cell 37*:767-778 (1984). As discussed below, other such fusion proteins include the Caspase-14 fused to Fc at the N- or C-terminus.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the Caspase-14 protein. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions, which may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the Caspase-14 protein or portions thereof. Also especially preferred in this regard are conservative substitutions.

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence shown as residues 96-270 in SEQ ID NO:2; (d) a nucleotide sequence encoding the polypeptide having the amino acid sequence shown as residues 289-377 in SEQ ID NO:2; (e) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No.209039; and (f) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d) or (e).

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding the Caspase-14 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding a Caspase-14 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted

with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 or to the nucleotide sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the length of the subject nucleotide sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only bases outside the 5' and 3' bases of the subject

sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score. For example, a 90 base subject sequence is aligned to a 100 base query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 bases at 5' end. The 10 unpaired bases represent 10% of the sequence (number of bases at the 5' and 3' ends not matched/total number of bases in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 bases were perfectly matched the final percent identity would be 90%. In another example, a 90 base subject sequence is compared with a 100 base query sequence. This time the deletions are internal deletions so that there are no bases on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only bases 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to made for the purposes of the present invention.

The present application is directed to nucleic acid molecules at least 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or to the nucleic acid sequence of the deposited cDNA, irrespective of whether they encode a polypeptide having Caspase-14 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having Caspase-14 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having Caspase-14 activity include, *inter alia,* (1) isolating the Caspase-14 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the Caspase-14 gene, as described in Verma *et al., Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and Northern Blot analysis for detecting Caspase-14 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in SEQ ID NO:1 or to a nucleic acid sequence of the deposited cDNA which do, in fact, encode a polypeptide having Caspase-14 protein activity. By "a polypeptide having Caspase-14 activity" is intended polypeptides exhibiting Caspase-14 activity in a particular biological assay.

The activity of purified or expressed Caspase-14 is tested by methods known in the art. One such method calls for stimulating macrophages with LPS to induce the expression of pre-IL1-Beta and then treating with Caspase-14 and ATP. Mature IL1-Beta levels in the medium are measured by enzyme-linked immunosorbent assay (ELISA), as described in Li et al. (1995) *Cell* **80**:401, incorporated herein by reference in its entirety.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 95%, 96%, 97%, 98%, or 99% identical to a nucleic acid sequence of the deposited cDNA or a nucleic acid sequence shown in SEQ ID NO:1 will encode a polypeptide "having Caspase-14 protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having Caspase-14 protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.,* "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Vectors and Host Cells

The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of Caspase-14 polypeptides or fragments thereof by recombinant techniques.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac, trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells;

insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods In Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL5-receptor has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson et *al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

The Caspase-14 protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction

chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Caspase-14 Polypeptides and Fragments

The invention further provides an isolated Caspase-14 polypeptide having the amino acid sequence encoded by the deposited cDNA, or the amino acid sequence in SEQ ID NO:2, or a peptide or polypeptide comprising a portion of the above polypeptides.

It will be recognized in the art that some amino acid sequences of the Caspase-14 polypeptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the Caspase-14 polypeptide which show substantial Caspase-14 polypeptide activity or which include regions of Caspase-14 protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990).

Based on the x-ray crystal structure of ICE, several amino acid residues critical for binding and catalysis have been identified. See, for example, Walker et al., Cell 78:343 (1994). These residues include the catalytic diad Cys-258 and His-210, and Gly-211 that stabalizes the tetrahydral intermediate. Arg-152, Gln-256, Arg-314, and Ser-320 form the binding pocket for the S1 substrate. These seven residues are conserved in all caspases thus far characterized including Caspase-14. Furthermore, the QAC(R/Q/G)G motif conserved in all caspases is conserved in Caspase-14 (residues 256-260 in SEQ ID NO:2). Thus, polypeptide variants of the invention preferrably include those that retain the above-described residues which are conserved among all caspases.

To map caspase-8 processing sites within Caspase-14, potential aspartate cleavage sites were mutated and tested as caspase-8 substrates. Productive cleavage was found to require Asp-289 as alteration of this residue abolished processing, data not shown. Therefore, Caspase-14 must be cleaved by caspase-8 following Asp-289 to yield an active heterodimeric enzyme.

Notably, this aspartate residue is found in the sequence context LEED (residues 286 to 289 in SEQ ID NO:2) which is the preferred substrate for caspase-8 cleavage. Accordingly, Caspase-14 polypeptide variants which include amino acids in the region of 286 to 289 preferrably retain an Asp residue at the position corresponding to Asp-289 in the full-length Caspase-14 polypeptide shown as SEQ ID NO:2.

Thus, the fragment, derivative or analog of the polypeptide of SEQ ID NO:2, or that encoded by the deposited cDNA, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the Caspase-14 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al., Clin. Exp. Immunol.* 2:331-340 (1967); Robbins *et al., Diabetes 36*:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems 10*:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Van Ostade *et al., Nature 361*:266-268 (1993) describes certain mutations resulting in selective binding of TNF-mutants to only one of the two known types of TNF receptors. Thus, the Caspase-14 of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine
	Tryptophan
	Tyrosine
Hydrophobic	Leucine
	Isoleucine
	Valine
Polar	Glutamine
	Asparagine
Basic	Arginine
	Lysine
	Histidine
Acidic	Aspartic Acid
	Glutamic Acid
Small	Alanine
	Serine
	Threonine
	Methionine
	Glycine

Amino acids in the Caspase-14 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science 244*:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al., J. Mol. Biol. 224*:899-904 (1992) and de Vos *et al. Science 255*:306-312 (1992)).

The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for

purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell. For example, a recombinantly produced version of the Caspase-14 polypeptide can be substantially purified by the one-step method described in Smith and Johnson, *Gene 67*:31-40 (1988).

The polypeptides of the present invention include the polypeptide encoded by the deposited cDNA; a polypeptide comprising amino acids about 1 to about 377 in SEQ ID NO:2; a polypeptide comprising amino acids about 2 to about 377; a polypeptide comprising amino acids from about 96 to about 270; and a polypeptide comprising amino acids from about 289 to about 377, as well as polypeptides which are at least 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to those described above and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of the Caspase-14 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the Caspase-14 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 or to the amino acid sequence encoded by deposited cDNA clone can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

Preferrably a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid

sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, (indels) or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in Table 1 or to the amino acid sequence encoded by deposited DNA clone can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. (1990) 6:237-245). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number

of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to made for the purposes of the present invention.

The polypeptide of the present invention is useful as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide described herein. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al., Proc. Natl. Acad. Sci. USA 81*:3998- 4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A. (1983) Antibodies that react with predetermined sites on proteins. *Science 219:*660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al., Cell 37*:767-778 (1984) at 777.

Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate Caspase-14 -specific antibodies include: a polypeptide comprising amino acid residues from about 35 to about 71 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 79 to about 99 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 110 to about 138 in SEQ ID NO:2, a polypeptide comprising amino acid residues from about 173 to about 202 in SEQ ID NO:2, a polypeptide comprising amino acid residues from about 221 to about 250 in SEQ ID NO:2, a polypeptide comprising amino acid residues from about 259 to about 297 in SEQ ID NO:2, a polypeptide comprising amino acid residues from about 305 to about 318 in SEQ ID NO:2, a polypeptide comprising amino acid residues from about 343 to about 370 in SEQ ID NO:2. As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the Caspase-14 protein (see Figure 2).

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R. A. (1985) General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA 82*:5131-5135. This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

As one of skill in the art will appreciate, Caspase-14 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al., Nature 331*:84- 86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric Caspase-14 protein or protein fragment alone (Fountoulakis *et al., J. Biochem 270*:3958-3964 (1995)).

Cancer Diagnosis and Prognosis

Breast carcinoma cells (MCF7) and Embryonic kidney cells (293) were transfected with an expression vector encoding the full-length Caspase-14 polypeptide and assayed for apoptosis. Like other caspases, Caspase-14 was able to induce cell death. However, unlike caspase-4 and -5, removal of the prodomain was not necessary to induce apoptosis. Furthermore, apoptosis induced by Caspase-14 was efficiently blocked by virally encoded caspase inhibitors p35 and CrmA.

To address whether Caspase-14 is productively processed by caspase-8 (the apical caspase involved in proximal death receptor signalling), Caspase-14 was incubated with caspase-8 and the emergence of active Caspase-14 was assessed by reaction with biotinylated-YVAD cmk

which covalently binds the catalytic cysteine within the large subunit of proteolytically competent (active) caspases. Caspase-8 processing of Caspase-14 led to the generation of two subunits. One of the subunits was the prodomain plus the large catalytic subunit (pro + large) and the other was the small catalytic subunit. This is similar to the activation of caspase-1 in which the p45 zymogen must initially be processed to a stable p35 pro + large subunit. Further processing, namely cleavage between the pro and large subunit, is highly dilutional sensitive and very inefficient in vitro such that in vitro translated zymogens do not undergo complete processing. Given the low concentration of in vitro-translated Caspase-14, it was not surprising that caspase-8 processed it only to the pro+large and small subunits. Regardless, caspase-8 processed Caspase-14 was efficiently labelled with biotinylated YVAD, indicative of generation of active Caspase-14.

It is believed that certain tissues in mammals with cancer express significantly depressed levels of the Caspase-14 protein and mRNA encoding the Caspase-14 protein when compared to a corresponding "standard" mammal, i.e., a mammal of the same species not having the cancer. Further, it is believed that depressed levels of the Caspase-14 protein can be detected in certain body fluids (e.g., sera, plasma, urine, and spinal fluid) from mammals with cancer when compared to sera from mammals of the same species not having the cancer. Thus, the invention provides a diagnostic method useful during tumor diagnosis, which involves assaying the expression level of the gene encoding the Caspase-14 protein in mammalian cells or body fluid and comparing the gene expression level with a standard Caspase-14 gene expression level, whereby a decrease in the gene expression level over the standard is indicative of certain tumors.

Where a tumor diagnosis has already been made according to conventional methods, the present invention is useful as a prognostic indicator, whereby patients exhibiting depressed Caspase-14 gene expression will experience a worse clinical outcome relative to patients expressing the gene at a higher level.

By "assaying the expression level of the gene encoding the Caspase-14 protein" is intended qualitatively or quantitatively measuring or estimating the level of the Caspase-14 protein or the level of the mRNA encoding the Caspase-14 protein in a first biological sample either directly (e.g., by determining or estimating absolute protein level or mRNA level) or relatively (e.g., by comparing to the Caspase-14 protein level or mRNA level in a second biological sample).

Preferably, the Caspase-14 protein level or mRNA level in the first biological sample is measured or estimated and compared to a standard Caspase-14 protein level or mRNA level, the standard being taken from a second biological sample obtained from an individual not having the cancer. As will be appreciated in the art, once a standard Caspase-14 protein level or mRNA level is known, it can be used repeatedly as a standard for comparison.

By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source which contains Caspase-14 protein or mRNA. Biological

samples include mammalian body fluids (such as sera, plasma, urine, synovial fluid and spinal fluid) which contain secreted Caspase-14 protein, and particularly fibroblast tissue.

The present invention is useful for detecting cancer in mammals. In particular the invention is useful during diagnosis of the of following types of cancers in mammals: breast, ovarian, prostate, bone, liver, lung, pancreatic, and skin. Preferred mammals include monkeys, apes, cats, dogs, cows, pigs, horses, rabbits and humans. Particularly preferred are humans.

Total cellular RNA can be isolated from a biological sample using the single-step guanidinium-thiocyanate-phenol-chloroform method described in Chomczynski and Sacchi, *Anal. Biochem. 162:*156-159 (1987). Levels of mRNA encoding the Caspase-14 protein are then assayed using any appropriate method. These include Northern blot analysis (Harada *et al., Cell 63:*303-312 (1990)), S1 nuclease mapping (Fujita *et al., Cell 49:*357- 367 (1987)), the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR) (Makino *et al., Technique 2:*295-301 (1990)), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Assaying CASPASE-14 protein levels in a biological sample can occur using antibody-based techniques. For example, Caspase-14 protein expression in tissues can be studied with classical immunohistological methods (Jalkanen, M., *et al., J. Cell. Biol. 101:*976-985 (1985); Jalkanen, M., *et al., J. Cell . Biol. 105:*3087-3096 (1987)).

Other antibody-based methods useful for detecting Caspase-14 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as, Glucose oxidase, and radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Caspase-14 Binding Molecules and Assays

This invention also provides a method for identification of molecules, such as receptor molecules, that bind Caspase-14. Genes encoding proteins that bind Caspase-14, such as receptor proteins, can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting. Such methods are described in many laboratory manuals such as, for instance, Coligan et al., *Current Protocols in Immunology 1(2):*Chapter 5 (1991).

For instance, expression cloning may be employed for this purpose. To this end polyadenylated RNA is prepared from a cell responsive to Caspase-14, a cDNA library is created from this RNA, the library is divided into pools and the pools are transfected individually into cells that are not responsive to Caspase-14. The transfected cells then are exposed to labeled Caspase-14. (Caspase-14 can be labeled by a variety of well-known techniques including standard methods of radio-iodination or inclusion of a recognition site for a site-specific protein

kinase.) Following exposure, the cells are fixed and binding of Caspase-14 is determined. These procedures conveniently are carried out on glass slides.

Pools are identified of cDNA that produced Caspase-14-binding cells. Subpools are prepared from these positives, transfected into host cells and screened as described above. Using an iterative sub-pooling and re-screening process, one or more single clones that encode the putative binding molecule, such as a receptor molecule, can be isolated.

Alternatively a labeled ligand can be photoaffinity linked to a cell extract, such as a membrane or a membrane extract, prepared from cells that express a molecule that it binds, such as a receptor molecule. Cross-linked material is resolved by polyacrylamide gel electrophoresis ("PAGE") and exposed to X-ray film. The labeled complex containing the ligand-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing can be used to design unique or degenerate oligonucleotide probes to screen cDNA libraries to identify genes encoding the putative receptor molecule.

Polypeptides of the invention also can be used to assess Caspase-14 binding capacity of Caspase-14 binding molecules, such as receptor molecules, in cells or in cell-free preparations.

Agonists and Antagonists - Assays and Molecules

The invention also provides a method of screening compounds to identify those which enhance or block the action of Caspase-14, such as its interaction with CASPASE-14-binding molecules such as receptor molecules. An agonist is a compound which increases the natural biological functions of Caspase-14 or which functions in a manner similar to Caspase-14, while antagonists decrease or eliminate such functions.

For example, a cellular compartment, such as a membrane or a preparation thereof, may be prepared from a cell that expresses a molecule that binds Caspase-14, such as a molecule of a signaling or regulatory pathway modulated by Caspase-14. The preparation is incubated with labeled Caspase-14 in the absence or the presence of a candidate molecule which may be a Caspase-14 agonist or antagonist. The ability of the candidate molecule to bind the binding molecule is reflected in decreased binding of the labeled ligand. Molecules which bind gratuitously, i.e., without inducing the effects of Caspase-14 on binding the Caspase-14 binding molecule, are most likely to be good antagonists. Molecules that bind well and elicit effects that are the same as or closely related to Caspase-14 are agonists.

Caspase-14-like effects of potential agonists and antagonists may be measured, for instance, by determining activity of a second messenger system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of Caspase-14 or molecules that elicit the same effects as Caspase-14. Second messenger systems that may be useful in this regard include but are not limited to proteolysis of downstream caspases.

Another example of an assay for Caspase-14 antagonists is a competitive assay that combines Caspase-14 and a potential antagonist with membrane-bound Caspase-14 receptor molecules or recombinant Caspase-14 receptor molecules under appropriate conditions for a competitive inhibition assay. Caspase-14 can be labeled, such as by radioactivity, such that the number of Caspase-14 molecules bound to a receptor molecule can be determined accurately to assess the effectiveness of the potential antagonist.

The agonist may be employed for instance to enhance the action of Caspase-14 polypeptides.

The antagonists may be employed for instance to inhibit the action of Caspase-14 polypeptides, for example, in the treatment of Alzheimer's disease, Parkinson's disease, rheumatoid arthritis, septic shock, sepsis, stroke, CNS inflammation, osteoporosis, ischemia, reperfusion injury, cell death associated with cardiovascular disease, polycystic kidney disease, apoptosis of endothelial cells in cardiovascular disease, degenerative liver disease, MS and head injury damage.

The agonists and antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

Therapeutics

The novel mammalian effector designated Caspase-14 of the present invention, is a catalytically active structural homologue of Caspase-4, and other caspases, that enhance TNFR-1, TRAIL and CD-95 induced apoptosis. Apoptosis is a useful regulator of cell growth and proliferation. Thus, Caspase-14 is useful in the treatment of cancers, particularly of the skin. Such cancers include melanomas.

Modes of administration

It will be appreciated that conditions caused by a decrease in the standard or normal level of Caspase-14 activity in an individual, can be treated by administration of Caspase-14 protein. Thus, the invention further provides a method of treating an individual in need of an increased level of Caspase-14 activity comprising administering to such an individual a pharmaceutical composition comprising an effective amount of an isolated Caspase-14 polypeptide of the invention, particularly a mature form of the Caspase-14, effective to increase the Caspase-14 activity level in such an individual.

As a general proposition, the total pharmaceutically effective amount of Caspase-14 polypeptide administered parenterally per dose will be in the range of about 1 μg/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the Caspase-14 polypeptide is typically administered at a dose rate of about 1 μg/kg/hour to about 50

µg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Pharmaceutical compositions containing the Caspase-14 of the invention may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), bucally, or as an oral or nasal spray. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Chromosome Assays

The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a Caspase-14 protein gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA then is used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, in some cases, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3 untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with probes from the cDNA as short as 50 or 60 bp. For a review of this technique, see Verma *et al., Human Chromosomes: A Manual Of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance In Man*, available on-line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Drug Screening

Caspase 14, or biologically or immunologically active fragments thereof, are used for screening compounds in any of a variety of drug screening techniques. The CASPASE-14 polypeptide or fragment employed in such a test is either free in solution, affixed to a solid support, borne on a cell surface or located intracellularly. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing CASPASE-14 or a fragment thereof. Drugs are screened against such transformed cells in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One may measure, for example, the formation of complexes between CASPASE-14 and the agent being tested. Alternatively, one can examine the diminution in complex formulation between CASPASE-14 and its target cell, the monocyte or macrophage, caused by the agent being tested.

Thus, the present invention provides methods of screening for drugs, natural inhibitors or any other agents which can affect inflammation and disease. These methods comprise contacting such an agent with a CASPASE-14 polypeptide or fragment thereof and assaying 1) for the presence of a complex between the agent and the CASPASE-14 polypeptide or fragment, or 2) for the presence of a complex between the CASPASE-14 polypeptide or fragment and the cell, by methods well known in the art. In such competitive binding assays, the CASPASE-14 polypeptide or fragment is typically labeled. After suitable incubation, free CASPASE-14 polypeptide or fragment is separated from that present is bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular agent to bind the CASPASE-14 or to interfere with the CASPASE-14/cell complex and agent complex.

Another technique for drug screening provides high throughout screening for compounds having suitable binding affinity to the CASPASE-14 polypeptide and is described in detail in European Patent Application 84/03564, published on September 13, 1984, incorporated herein by reference. Briefly stated, a plurality of different peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with CASPASE-14 polypeptide and washed. Bound CASPASE-14 polypeptide is then detected by methods well known in the art. Purified CASPASE-14 can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be used to capture the peptide and immobilize it on the solid support.

The invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding CASPASE-14 specifically compete with a test

compound for binding to CASPASE-14 polypeptides or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with CASPASE-14.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Examples

Example 1: Expression and Purification of Caspase-14 in E. coli

The bacterial expression vector pQE60 is used for bacterial expression in this example. (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin sold by QIAGEN, Inc., *supra*, and suitable single restriction enzyme cleavage sites. These elements are arranged such that an inserted DNA fragment encoding a polypeptide expresses that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide.

The DNA sequence encoding the desired portion of the Caspase-14 protein is amplified from the deposited cDNA clone using PCR oligonucleotide primers which anneal to the amino terminal sequences of the desired portion of the Caspase-14 protein and to sequences in the deposited construct 3' to the cDNA coding sequence. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning the protein, the 5' primer has the sequence 5' CGC CCATGG CTGAAGACAAACACAAC 3' (SEQ ID NO:3) containing the underlined *Nco*I restriction site followed by 17 (i.e., 47-63) nucleotides complementary to the amino terminal coding sequence of the Caspase-14 sequence in Figure 1. One of ordinary skill in the art would appreciate, of course, that the point in the protein coding sequence where the 5' primer begins may be varied to amplify a DNA segment encoding any desired portion of the complete protein in a shorter or longer form. The 3' primer has the sequence 5' CGC AAG CTT AACATGGATGCTGTGCTG 3' (SEQ ID NO:4) containing the underlined *Hind*III restriction site followed by 18 (i.e., 1230-1247) nucleotides complementary to the 3' end of the coding sequence immediately before the stop codon in the Caspase-14 DNA sequence in Figure 1, with the coding sequence aligned with the restriction site so as to maintain its reading frame with that of the six His codons in the pQE60 vector.

The amplified Caspase-14 DNA fragment and the vector pQE60 are digested with *NcoI/HindIII* and the digested DNAs are then ligated together. Insertion of the Caspase-14 DNA into the restricted pQE60 vector places the Caspase-14 protein coding region downstream from the IPTG-inducible promoter and in-frame with an initiating AUG and the six histidine codons.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described in Sambrook *et al.*, *Molecular Cloning: a Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kan^r"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing Caspase-14 protein, is available commercially from QIAGEN, Inc., *supra.* Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. Isopropyl-b-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

The cells are then stirred for 3-4 hours at 4 C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the Caspase-14 protein is loaded onto a nickel-nitrilo-tri-acetic acid ("NiNTA") affinity resin column (available from QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the NI-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist, 1995, QIAGEN, Inc., *supra).* Briefly the supernatant is loaded onto the column in 6 M guanidine-HCl, pH8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH8, then washed with 10 volumes of 6 M guanidine-HCl pH6, and finally the Caspase-14 is eluted with 6 M guanidine-HCl, pH5.

The purified protein is then renatured by dialyzing it against phosphatebuffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. The recommended conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins can be eluted by the addition of 250 mM immidazole. Immidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH6 buffer plus 200 mM NaCl. The purified protein is stored at 4 C or frozen at -80 C.

Example 2: Cloning and Expression of Caspase-14 protein in a Baculovirus Expression System

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding the complete protein into a baculovirus to express the Caspase-14 protein, using standard methods as described in Summers *et al., A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures,* Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites such as *BamHI* and *Asp*718. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak Drosophila promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of the vector above, such as pAc373, pVL941 and pAcIM1, as one skilled in the art would readily appreciate, as long as the construct provides appropriately located signals for transcription, translation, secretion and the like, including a signal peptide and an in-frame AUG as required. Such vectors are described, for instance, in Luckow *et al., Virology 170*:31-39.

The cDNA sequence encoding the full length Caspase-14 protein in the deposited clone, including the AUG initiation codon shown in Figure 1 (SEQ ID NO:2), is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence 5' CGC GGA TCC GCCATCATGGCTGAAGACAAACAC 3' (SEQ ID NO:5) containing the underlined *BamHI* restriction enzyme site, an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987), followed by 17 (i.e., 43-60) bases of the sequence of the complete Caspase-14 protein shown in Figure 1, beginning with the AUG initiation codon. The 3' primer has the sequence 5' CGC GGT ACCAACATGGATGCTGTGCTG 3' (SEQ ID NO:6) containing the underlined, *Asp*718 restriction site followed by 17 (1230-1247) nucleotides complementary to the 3' noncoding sequence in Figure 1.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with *BamHI* and *Asp*718 and again is purified on a 1% agarose gel.

The plasmid is digested with the restriction enzymes *BamHI* and *Asp*718 and optionally, can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.).

Fragment and the dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human Caspase-14 gene using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies containing the Caspase-14 gene fragment will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac Caspase-14.

Five μg of the plasmid pBac Caspase-14 is co-transfected with 1.0 μg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al., Proc. Natl. Acad. Sci. USA 84*:7413-7417 (1987). 1 μg of BaculoGold™ virus DNA and 5 μg of the plasmid pBac Caspase-14 are mixed in a sterile well of a microtiter plate containing 50 μl of serum-free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards, 10 μl Lipofectin plus 90 μl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27 C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27 C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, *supra.* An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g., Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 μl of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4 C. The recombinant virus is called V- Caspase-14.

To verify the expression of the Caspase-14 gene, Sf9 cells are grown in Grace's medium supplemented with 10% heat inactivated FBS. The cells are infected with the recombinant baculovirus V- Caspase-14 at a multiplicity of infection ("MOI") of about 2. Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Rockville, MD). If radiolabeled proteins are desired, 42 hours later, 5 μCi of ^{35}S-methionine and 5 μCi ^{35}S-cysteine (available from Amersham) are

added. The cells are further incubated for 16 hours and then they are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature protein and thus the cleavage point and length of the secretory signal peptide should one exist.

Example 3: Cloning and Expression of Caspase-14 in Mammalian Cells

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRS) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as PSVL and PMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Mammalian host cells that could be used include, human Hela 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV 1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy *et al., Biochem J.* 227:277-279 (1991); Bebbington *et al., Bio/Technology 10:*169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molec. Cell. Biol. 5*:438-447 (1985)) plus a fragment of the CMV-enhancer (Boshart *et al., Cell 41*:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites *BamH*I, *Xba*I and *Asp*718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Example 3(a): Cloning and Expression of Caspase-14 in COS Cells

The expression plasmid, pCaspase-14 HA, is made by cloning a cDNA encoding Caspase-14 into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc., San Diego, CA).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson *et al., Cell 37:*767-778 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the Caspase-14 is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The Caspase-14 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of Caspase-14 in *E. coli.* Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined *Sma*I site, a Kozak sequence, an AUG start codon and 16 bases of 5' coding region of the complete Caspase-14 has the following sequence: 5' CGCCCCGGGGCCATCATGGCTGAAGACAAACAC 3' (43-60) (SEQ ID NO:7). The 3' primer, containing the underlined *Xba*I site, a stop codon, and 18 bp of 3' coding sequence has the following sequence (at the 3' end): 5' CGC TCTAGA TCA AGC GTA GTC TGG GAC GTC GTA TGG GTAGTTGCCAGGAAAGAGGT 3' (1157-1173) (SEQ ID NO:8).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with *Sma*I and *Xba*I and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the Caspase-14-encoding fragment.

For expression of recombinant Caspase-14, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook *et*

al., Molecular Cloning: a Laboratory Manual, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of Caspase-14 by the vector.

Expression of the Caspase-14-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow *et al., Antibodies: A Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing ^{35}S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson *et al.* cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

Example 3(b): Cloning and Expression of Caspase-14 in CHO Cells

The vector pC4 is used for the expression of Caspase-14 protein. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., 1978, *J Biol. Chem. 253*:1357-1370, Hamlin, J. L. and Ma, C. 1990, *Biochem. et Biophys. Acta, 1097*:107-143, Page, M. J. and Sydenham, M.A. 1991, *Biotechnology 9*:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains for expressing the gene of interest the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen *et al., Molec. Cell. Biol. 5*:438-447 (1985)) plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart *et al., Cell 41*:521-530 (1985)). Downstream of the promoter are *BamHI*, *XbaI*, and *Asp*718 restriction enzyme cleavage sites that allow integration of the genes. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of

the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the Caspase-14 in a regulated way in mammalian cells (Gossen, M., & Bujard, H. 1992, *Proc. Natl. Acad. Sci. USA 89*: 5547-5551). For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes *BamHI* and *Asp*718 and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete Caspase-14 protein is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene. The 5' primer has the sequence 5' CGC GGA TCC GCCATCATGGCTGAAGACAAACAC 3' (SEQ ID NO:9) containing the underlined *Bam*I restriction enzyme site, an efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol. 196*:947-950 (1987), followed by 17 (i.e., 43-60) bases of the sequence of the complete Caspase-14 protein shown in Figure 1, beginning with the AUG initiation codon. The 3' primer has the sequence 5' CGC GGT ACCAACATGGATGCTGTGCTG 3' (SEQ ID NO:10) containing the underlined, *Asp*718 restriction site followed by 17 (1230-1247) nucleotides complementary to the 3' noncoding sequence in Figure 1.

The amplified fragment is digested with the endonucleases *BamHI* and *Asp*718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. 5 µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSV2-neo using lipofectin (Felgner *et al., supra).* The plasmid pSV2neo contains a dominant selectable marker, the neo gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of metothrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-

well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 μM, 20 μM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reverse phase HPLC analysis.

Example 4: Tissue distribution of Caspase-14 mRNA expression

Northern blot analysis is carried out to examine Caspase-14 gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the Caspase-14 protein (SEQ ID NO:1) is labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN- 100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for Caspase-14 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) can be obtained from Clontech and are examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70 C overnight, and films developed according to standard procedures.

Experiments performed substantially as above revealed that Caspase-14 is expressed constitutively in a variety of human tissues. Caspase-14 was highly expressed in HeLa cells, but not in transformed hematopoietic cell lines including Burkitt's lymphoma, Raji cells or im promyelocytic leukemia HL-60 cells.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line 10

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution American Type Culture Collection ("ATCC")

Address of depositary institution *(including postal code and country)*
10801 University Boulevard
Manassas, Virginia 20110-22009
United States of America

Date of deposit	Accession Number
15 MAY 1997	209039

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

What Is Claimed Is:

1. An isolated polynucleotide comprising a nucleic acid sequence selected from the group consisting of:

(a) a nucleic acid sequence encoding the polypeptide shown as amino acid residues 1 to 377 in SEQ ID NO:2;

(b) a nucleic acid sequence encoding the polypeptide shown as amino acid residues 2 to 377 in SEQ ID NO:2;

(c) a nucleic acid sequence encoding the polypepetide shown as amino acid residues 96 to 270 in SEQ ID NO:2;

(d) a nucleic acid sequence encoding the polypeptide shown as amino acid residues 289 to 377 in SEQ ID NO:2;

(e) a nucleic acid sequence encoding the polypeptide encoded by the human cDNA clone contained in ATCC Deposit No. 209039;

(f) a nucleic acid sequence encoding the polypeptide encoded by the human cDNA clone contained in ATCC Deposit No. 209039, wherein said polypeptide lacks an N-terminal methionine;

(g) a nucleic acid sequence at least 95% identical to the nucleic acid sequence of (a), (b), (c), (d), (e) or (f);

(h) a nucleic acid sequence which hybridizes under stringent conditions to the human cDNA contained in ATCC Deposit No. 209039 or to a polynucleotide consisting of the complement of the nucleic acid sequence shown as SEQ ID NO:1;

(i) a nucleic acid sequence encoding a biologically active fragment of the polypeptide shown as amino acid residues 1 to 377 of SEQ ID NO:2 or the polypeptide encoded by the human cDNA contained in ATCC Deposit No. 209039;

(j) a nucleic acid sequence which encodes a biologically active fragment of the polypeptide shown as amino acid residues 1 to 377 of SEQ ID NO:2 or the polypeptide encoded by the human cDNA contained in ATCC Deposit No. 209039 wherein said polypeptide has at least one conservative substitution; and

(k) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k).

2. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1.

3. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1 encoding the Caspase-14 polypeptide having the amino acid sequence in SEQ ID NO:2.

4. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 209039.

5. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the Caspase-14 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 209039.

6. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c) (d) or (e) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

7. An isolated nucleic acid molecule comprising a polypeptide which encodes the amino acid sequence of an epitope-bearing portion of Caspase-14 polypeptide having an amino acid sequence in (a), (b), (c), (d) or (e) of claim 1.

8. The isolated nucleic acid molecule of claim 6, which encodes an epitope-bearing portion of Caspase-14 polypeptide selected from the group consisting of: a polypeptide comprising amino acid residues from 35 to 71, from 79 to 99, from 110 to 138, from 173 to 202, from 221 to 250, from 259 to 297, from 305 to 318, and from 343 to 370, all as shown in SEQ ID NO:2.

9. A method for making a recombinant vector comprising inserting the isolated polynucleotide of claim 1 into a vector.

10. A recombinant vector produced by the method of claim 9.

11. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 10 into a host cell.

12. A recombinant host cell produced by the method of claim 11.

13. A recombinant method for producing a Caspase-14 polypeptide comprising culturing the recombinant host cell of claim 12 under conditions such that said polypeptide is expressed and recovering said polypeptide.

14. An isolated Caspase-14 polypeptide having an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) amino acid residues 1 to 377 in SEQ ID NO:2;

(b) amino acid residues 2 to 377 in SEQ ID NO:2;

(c) amino acid residues 96 to 270 in SEQ ID NO:2;

(d) amino acid residues 289 to 377 in SEQ ID NO:2;

(e) the amino acid sequence of the polypeptide encoded by the human cDNA clone contained in ATCC Deposit No. 209039;

(f) the amino acid sequence of the polypeptide encoded by the human cDNA clone contained in ATCC Deposit No. 209039, wherein said polypeptide lacks an N-terminal methionine;

(g) an amino acid sequence at least 95% identical to the amino acid sequence of (a), (b), (c), (d), (e) or (f);

(h) the amino acid sequence of a biologically active fragment of the polypeptide shown as amino acid residues 1 to 377 of SEQ ID NO:2, or the amino acid sequence of a biologically active fragment of the polypeptide encoded by the human cDNA contained in ATCC Deposit No. 209039;

(i) the amino acid sequence of (h) having at least one conservative substitution; and

(j) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e) or (f).

15. An isolated polypeptide comprising an epitope bearing portion of the Caspase-14 protein, wherein said portion is selected from the group consisting of: a polypeptide comprising amino acid residues 35 to 71, 79 to 99, 110 to 138, 173 to 202, 221 to 250, 259 to 297, 305 to 318, and 343 to 370, all of SEQ ID NO:2.

16. The isolated polypeptide of claim 14, which is produced or contained in a recombinant host cell.

17. The isolated polypeptide of claim 16, wherein said recombinant host cell is mammalian.

FIGURE 1

```
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Ser Ile Lys Ala Pro Glu Glu Thr Val Ala Gly Pro Asp Glu Ser Val
 85                  90                  95                 100

gga tct gca gct acc ctc aag ctt tgc cct cat gaa gaa ttc ctg aaa   390
Gly Ser Ala Ala Thr Leu Lys Leu Cys Pro His Glu Glu Phe Leu Lys
                105                 110                 115

ctg tgt aaa gaa agg gct gga gag atc tat cca ata aag gag aga aag   438
Leu Cys Lys Glu Arg Ala Gly Glu Ile Tyr Pro Ile Lys Glu Arg Lys
            120                 125                 130

gac cgc act cgt ctg gct ctc atc ata tgc aac aca gag ttt gat cat   486
Asp Arg Thr Arg Leu Ala Leu Ile Ile Cys Asn Thr Glu Phe Asp His
        135                 140                 145

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    150                 155                 160

ctg ctt gag ggt ctt ggc tac act gtg gaa gtg gaa gag aaa ctc aca   582
Leu Leu Glu Gly Leu Gly Tyr Thr Val Glu Val Glu Glu Lys Leu Thr
165                 170                 175                 180

gcc agg gac atg gaa tca gtg ctg tgg aaa ttt gct gca cgt gaa gag   630
Ala Arg Asp Met Glu Ser Val Leu Trp Lys Phe Ala Ala Arg Glu Glu
                185                 190                 195

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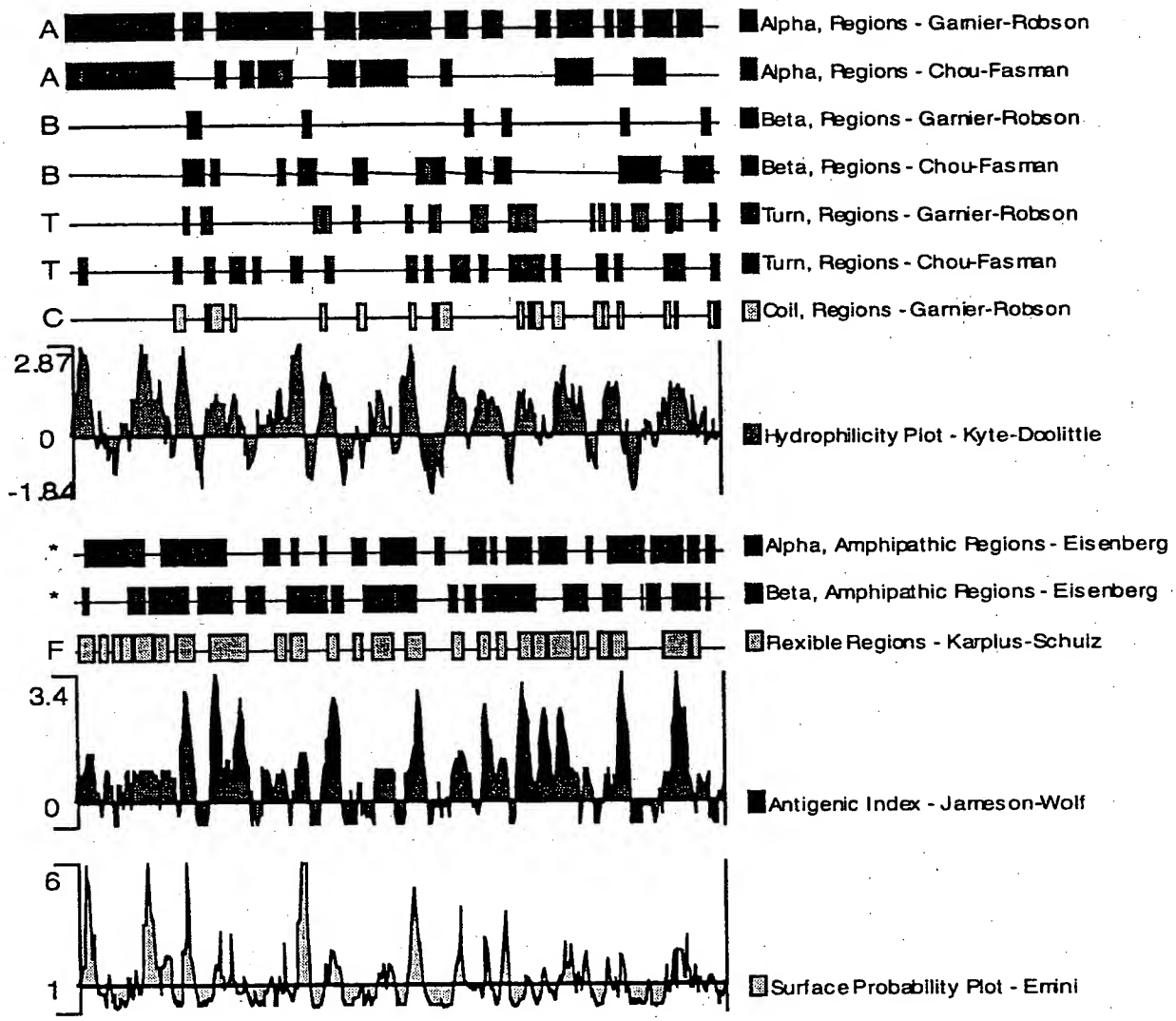
FIGURE 2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Coil, Regions - Garnier-Robson
Hydrophilicity Plot - Kyte-Doolittle
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Gly Ser Ala Ala Thr Leu Lys Leu Cys Pro His Glu Glu Phe Leu Lys
                105                 110                 115

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Leu Cys Lys Glu Arg Ala Gly Glu Ile Tyr Pro Ile Lys Glu Arg Lys
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Ser Leu Lys Asp Lys Pro Lys Val Ile Ile Val Gln Ala Cys Arg Gly
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Ala Gln Met Pro Thr Val Glu Arg Leu Ser Met Thr Arg Tyr Phe Tyr
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Glu Lys Leu Thr Ala Arg Asp Met Glu Ser Val Leu Trp Lys Phe Ala
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Ala Arg Glu Glu His Lys Ser Ser Asp Ser Thr Phe Leu Val Phe Met
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Arg Asn Cys Leu Ser Leu Lys Asp Lys Pro Lys Val Ile Ile Val Gln
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Ala Cys Arg Gly Ala Asn Arg Gly Glu Leu Trp Val Ser Asp Ser Pro
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Pro Ala Leu Ala Asp Ser Phe Ser Gln Ser Ser Glu Asn Leu Glu Asp
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Asp Ala Val Tyr Lys Thr His Val Glu Lys Asp Phe Ile Ala Phe Cys
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Ser Ser Thr Pro His Asn Val Ser Trp Arg Asp Ile Lys Lys Gly Ser
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Leu Phe Ile Thr Arg Leu Ile Thr Cys Phe Gln Lys Tyr Ala Trp Cys
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Cys His Leu Glu Glu Val Phe Arg Lys Val Pro Gln Ser Phe Glu Lys
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20452

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07H 21/04; C12N 15/00, 5/00, 1/14, 1/20, 9/48; C12Q 1/68
US CL :536/23.2, 24.3; 435/252.3, 254.11, 325, 320.1, 212, 6
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.2, 24.3; 435/252.3, 254.11, 325, 320.1, 212, 6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MUNDAY ET AL. Molecular Cloning and Pro-apoptotic Activity of ICE(rel)II and ICE(rel)III, Members of the ICE/CED-3 Family of Cysteine Proteases. J. Biol. Chem. June 1995, Vol. 270, pages 15870-15876, see entire document.	1-17
A	HENKART, P.A. ICE Family Proteases: Mediators of All Apoptotic Cell Death? Immunity. March 1996, Vol. 4, pages 195-201, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 DECEMBER 1998	Date of mailing of the international search report 27 JAN 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LISA J. HOBBS, PH.D. Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/20452

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS and STN (Bioscience and Patents Indexes): caspase#, interleukin converting enzyme#, ice, apoptosis, cysteine protease#, aspartic acid, specific###; N-Geneseq, GenBank, EST1-4, A-Geneseq, PIR, Swissprot: Seq. ID Nos.: 1 and 2.